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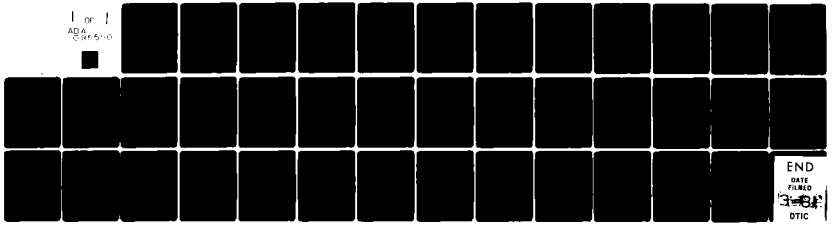

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STUDIES ON AMOEBIASIS (U)

FINAL REPORT

by

Murray Wittner, M.D., Ph.D. and Robert M. Rosenbaum, Ph.D.

December 1971
(For the period 1 September 1967 to 30 June 1971)

Supported by

U.S. ARMY MEDICAL RESEARCH & DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701
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Axenic strains of Entamoeba histolytica are being studied with regard to factors that might influence virulence and invasibility. Studies on the development of a defined growth medium have been undertaken. Ultrastructural features of the trophozoite are described.

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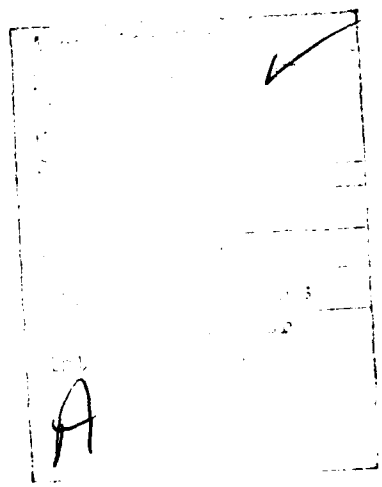

SUMMARY

Axenic strains of Entamoeba histolytica are being studied from the point of view of the intracellular control of virulence. Reassociation with various bacterial strains alters virulence and is correlated with hydrolytic enzymic as well as nucleic acid synthesis. A model system is utilized to study enzyme induction and its relation to increased virulence discussed.

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TABLE I

ELECTROPHORETIC COMPOSITION OF EQUINE SERUM PROTEIN

Fractions Obtained Following Ammonium Sulphate Fractionation

Fraction No.	$(NH_4)_2SO_4$ % Sat.	$\%N_2$	Protein components as % of total			
			γ globulins	β globulins	α globulins	albumins
I	28	12.2	82.4	17.6	0	0
II	35	13.6	70.6	29.4	0	0
III	43	12.4	51.8	21.8	6.4	0
IV	50	10.2	19.8	39.6	23.2	17.4
V	58	12.6	0	26.5	38.9	34.6
VI	64	12.8	0	22.0	26.2	51.8
VII	75	8.4	0	0	0	100.0

TABLE II

Acid Phosphatase Activity Following Induction with Substrate*

Induction Time	iPμg**	Mean μg	% increase
0 hours	93.7		
	90.1	90.8	
	88.7		
1 hour	97.3		
	99.0	99.3	9.36
	101.5		
2 hours	100.3		
	107.5	105.3	15.97
	108.1		
6 hours	197.4		
	205.3	210.9	132.27
	230.2		
12 hours	459.2		
	493.6	454.6	400.66
	411.0		

*liberation of inorganic phosphate after incubation at 37°C. for one hour.

** 3 separate determinations.

COMPOSITION OF MOD[illegible]BASIC MEDIUM *

TABLE III

Casamino acids (DIFCO)	10.0 grams
Lactalbumin hydrolysate (NBC)	5.0
Trypticase (BBL)	5.0
Oxoid	15.0
Glucose	5.0
L-Cysteine-HCl	0.75 grams
Mercaptosuccinic Acid	1.0
L-Ascorbic Acid	0.2
Na Cl	5.0
KH_2PO_4	0.6
K_2HPO_4 (Anhydrous)	0.8
H_2O (Distilled)	800 ml

pH adjusted to 7.1 with IN NaOH autoclaved 121° C for 12 min.

* 100 ml inactivated horse serum and 25 ml of vitamins (NCTC 109) are added aseptically. Agar 0.02 % (W/V) can be added.

TABLE IV

Comparative growth of axenic Entamoeba histolytica (Strain 301) in diaphasic media and in monophasic medium.

MEDIUM	INOCULUM/ml	# OF AMOEBAE IN 96 HOURS	% INCREASE
Diphasic-Diamond's	12,500	65,000	520
Diaphasic-CAA	12,500	76,250	610
Monophasic-CAA	12,500	68,750	550

TABLE V

Fate of H^3-thymidine labelled bacteria in previously axenic Entamoeba histolytica

Bacterial Feeding Time	DISINTEGRATION/MIN					
	Living		Heat-Killed		X-Ray Killed	
	NUC	CYTOPLASM	NUC	CYTOPLASM	NUC	CYTOPLASM
5	3	57	1	38	6	62
10	65	91	17	168	14	85
15	91	127	15	240	14	221
20	122	250	18	310	15	350
25	381	503	19	660	19	685
30	515	619	15	922	11	698

TABLE VI

Fate of H^3-uridine labelled bacteria in previously Axenic Entamoeba histolytica

Bacterial Feeding	Living		Heat Killed		X-Ray Killed	
Time	NUC	CYTOPLASM	NUC	CYTOPLASM	NUC	CYTOPLASM
5	17	8	3	56	32	101
10	19	127	15	227	8	199
15	22	593	55	778	11	403
20	11	1165	9	985	12	717
25	31	1530	28	1280	39	1503
30	6	1870	6	1410	20	2181

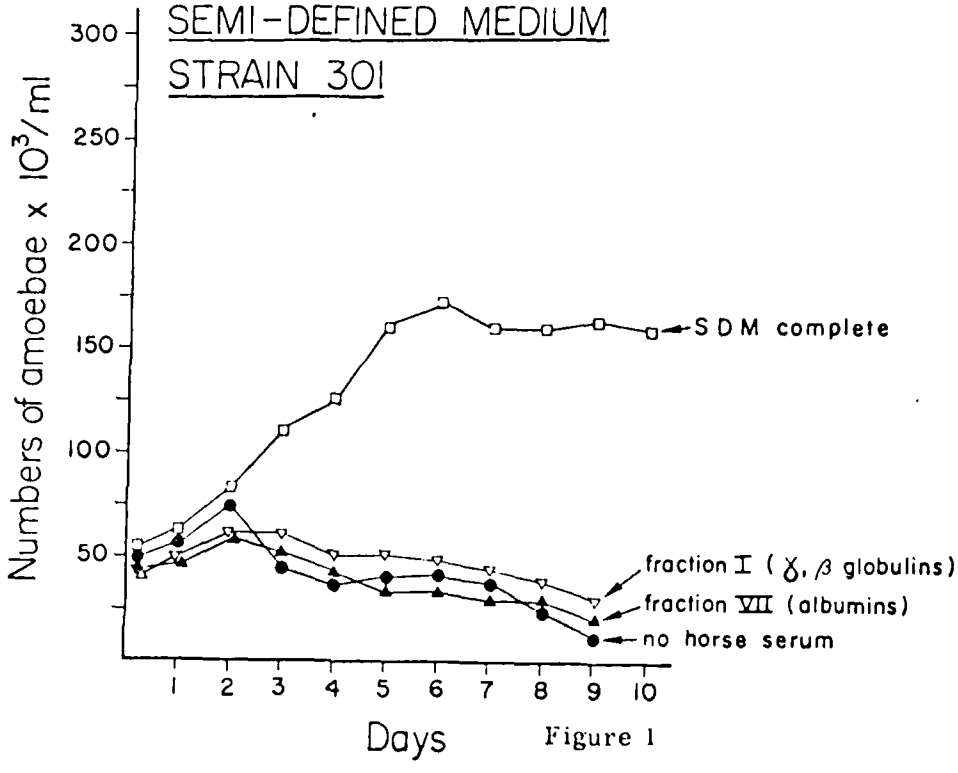
SEMI-DEFINED MEDIUM
STRAIN 301
Numbers of amoebae x 10^3/ml
300
250
200
150
100
50
1
2
3
4
5
6
7
8
9
10
Days
SDM complete
fraction I (γ, β globulins)
fraction VII (albumins)
no horse serum

Figure 1

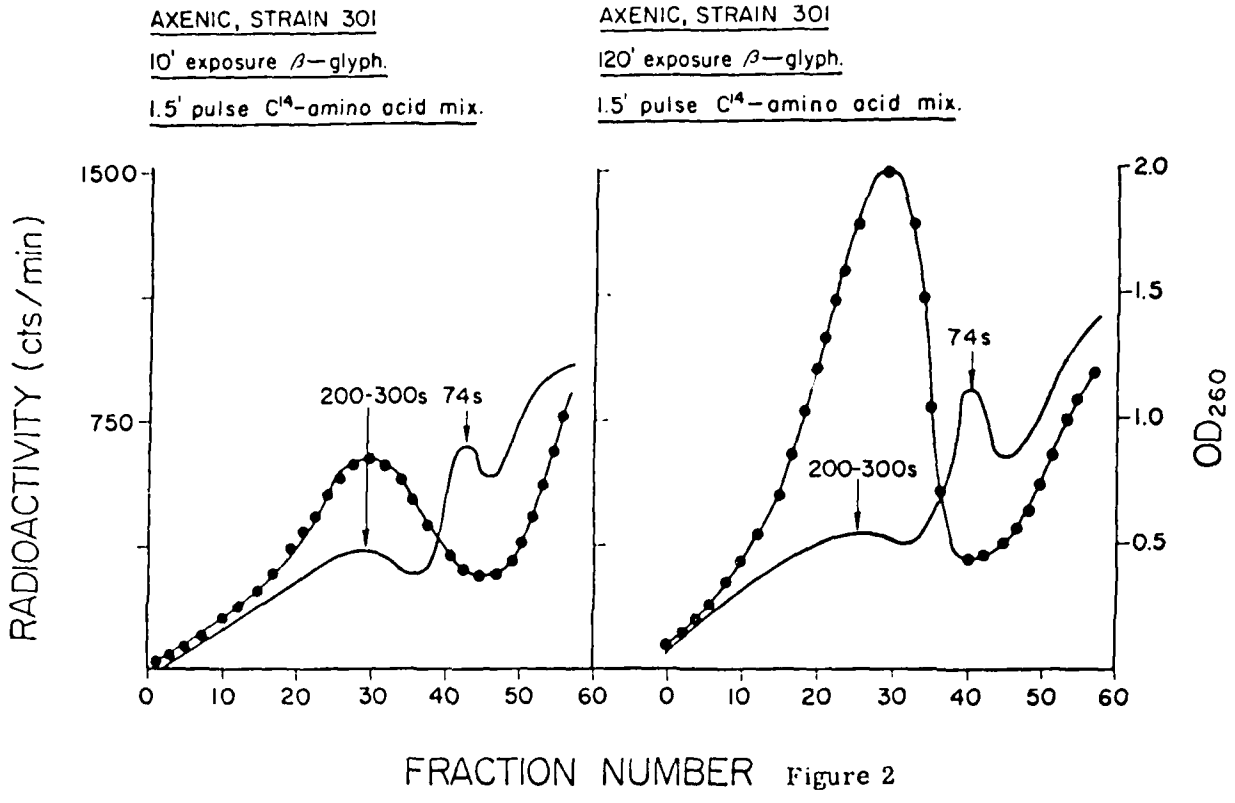
AXENIC, STRAIN 301
10' exposure β—glyph.
1.5' pulse C14—amino acid mix.
AXENIC, STRAIN 301
120' exposure β—glyph.
1.5' pulse C14—amino acid mix.
RADIOACTIVITY (cts/min)
1500
750
200-300s
74s
200-300s
74s
OD260
2.0
1.5
1.0
0.5
0
10
20
30
40
50
60
FRACTION NUMBER

Figure 2

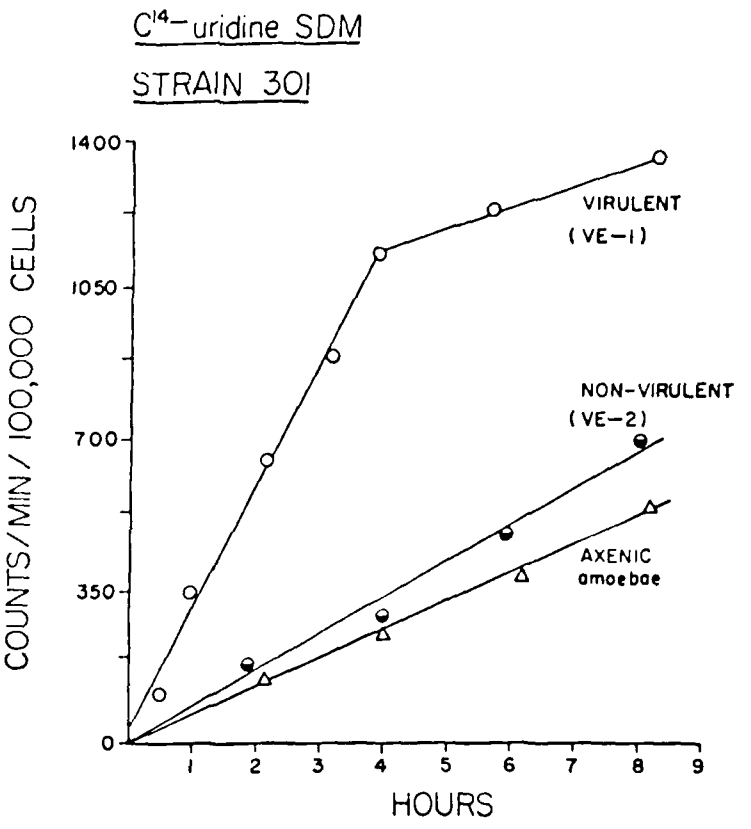
C14–uridine SDM
STRAIN 301
COUNTS/MIN/100,000 CELLS
1400
1050
700
350
0
1
2
3
4
5
6
7
8
9
HOURS
VIRULENT
(VE–1)
NON-VIRULENT
(VE–2)
AXENIC
amoebae

Figure 3

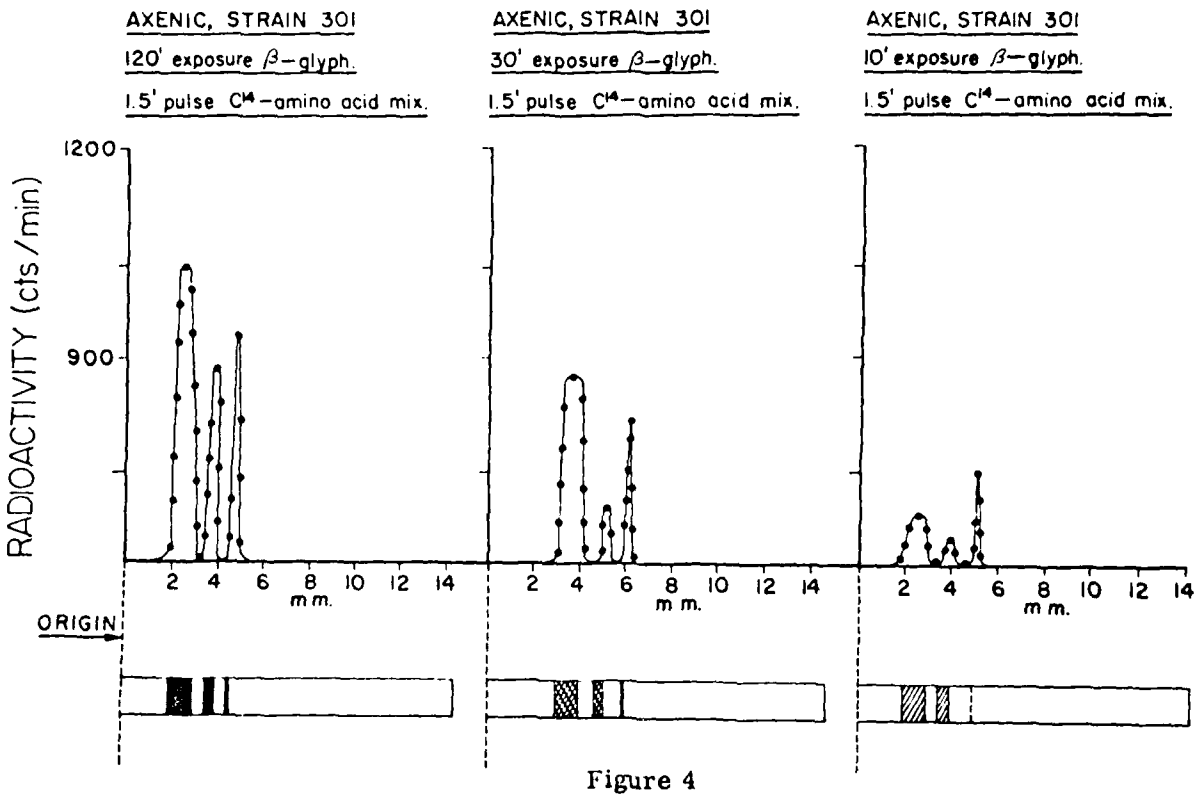
AXENIC, STRAIN 301
120' exposure β–glyph.
1.5' pulse C14–amino acid mix.
AXENIC, STRAIN 301
30' exposure β–glyph.
1.5' pulse C14–amino acid mix.
AXENIC, STRAIN 301
10' exposure β–glyph.
1.5' pulse C14–amino acid mix.
RADIOACTIVITY (cts/min)
1200
900
2 4 6 8 10 12 14
mm.
ORIGIN

Figure 4

Virulence of various strains of Entamoeba histolytica is variable and at times appears capricious. Those factors which modify virulence are ill-defined and are usually stated to be related in some manner to associated bacterial flora, host resistance or susceptibility, and the amoeba itself. Until recently it has been difficult to isolate these factors, one from the other, so that one might attempt to define the role of amoeba, associated bacteria and host in the control of virulence.

During the past several years our laboratory has been able to culture Entamoeba histolytica in axenic culture.[1] This achievement has permitted us to study E. histolytica without the interference or complications of any other associated organism or its metabolic products. Furthermore, utilizing axenic amoebae we have been able to manipulate the amoeba-bacterial-host relationship so that the contribution of each could be determined more readily.

I. Culture Medium

During the past year we have devoted considerable time and effort to attempting to improve our culture methods with the aim of obtaining a completely defined medium. During the past four years it has become perfectly clear that we could modify a variety of individual components in the medium and still support growth. Serum, however, has remained the one factor without which the medium was uniformly unsuccessful. We undertook, therefore, an ambitious project in which we fractionated horse serum and substituted these fractions for the regular horse serum component of the medium. Basically, we utilized the method of Keller and Block[2] in which we precipitated protein fractions from equine serum by step-wise increases in the quantity of ammonium sulfate. The ammonium sulfate was introduced into the aqueous solution of the serum proteins by dialysis of the serum against salt solutions of increasing strength. Table I shows the electrophoretic composition of the protein fractions. Figure 1 is presented as a typical example of the results obtained when serum fractions are substituted for whole horse serum. It is apparent that by themselves, or in combination, serum fractions will not support growth of the amoebae. Furthermore, the recombining of each fraction has not yielded satisfactory growth subsequently. This approach, therefore, has been most discouraging and has caused us to consider other means of attacking the problem. These are being developed and pursued at present.

II. Virulence

As we have described previously (see previous Progress Report), axenic amoebae inoculated into germ-free guinea pigs or rats fail to produce cecal or intestinal lesions. When reassociated with certain strains of Escherichia coli and then inoculated into germ-free animals lesions are obtained routinely. Bacterial reassociation has been noted to be correlated with heightened intracellular hydrolytic enzyme activity in the amoebae. Such enzyme activity could not be correlated solely with bacterial feeding in itself, although we attempted to determine this point. Since our earlier experiments suggested to us that bacteria acted as enzyme "inducers", we next added a simple organic phosphate substrate (Na_2-β-glycerophosphate) to axenic cultures and determined, by cytochemical staining technics, that there was enhanced

intracytoplasmic cytochemical localization of acid phosphatase. To prove this observation a 72 hour axenic culture was incubated for 0, 1, 2, 6, and 12 hours with 20 mg/ml Na_2-β-glycerophosphate. After washing in Earle's buffer, the amoebae were collected by centrifugation at 0°C. They were dounced 10 times in low ionic-strength buffer and a 1:44 cytoplasmic fraction (final dilution) was incubated with Na_2-β-glycerophosphate in an acetate buffer at pH 5.0. The reaction was stopped after one hour at 37°C. with ice-cold 10% TCA and the inorganic phosphate (iP) liberated was determined. Controls were run for iP in substrate-buffer and each cytoplasmic fraction. The densities were read in a Beckman DU Spectrophotometer at 660 mμ. Table II confirms our impression of the marked increase in enzyme activity when amoebae are incubated in the presence of substrate.

If these data were correct, we reasoned, enzyme "induction" or increased synthesis should be detectable by measuring new protein synthesis. We, therefore, proceeded to the next experiment in order to determine if substrate induction resulted in new protein synthesis.

After incubation from 10 minutes to 120 minutes in complete medium containing β-glycerophosphate, amoebae were placed in semidefined medium (SDM)[1] without serum and 1/40th amino acid content. Twenty minutes later 5μc/ml C^{14} Amino Acid mixture was added for a 1-1/2 minute pulse. Incorporation was stopped by pouring the cells and medium over frozen Earle's buffer. The cells were centrifuged at 0°C. and washed in ice-cold buffer. They were placed next in a low ionic-strength buffer for 10 minutes and dounced. Cytoplasm was separated from nuclei and made 0.5% with respect to sodium deoxycholate. The extract was layered on a 15-30% sucrose gradient, placed on an SW25.1 rotor and separated in a Spinco L2-65B Ultracentrifuge at 23,000 rpm for 90 minutes at 0°C. The gradient was tapped from the bottom by a needle. The material was propelled by a peristaltic finger pump through a continuous flow cell of a Gilford-modified Beckman DU Spectrophotometer set to read at a wave length of 260 mμ. One ml fractions were collected. Ten percent ice-cold TCA was added to each fraction which was then poured onto a Millipore filter (HA). The filter and the entrapped acid precipitable fraction were placed in Bray's solution and the radioactivity of each sample determined in a Packard low-background scintillation counter.

Figure 2 shows that following 120 minutes in an inducing-substrate, protein synthesis is markedly increased. There is nearly three times the uptake of C^{14} amino acid into nascent protein on polyribosomes. This is indicated by the sharp rise of radioactivity over the broad polyribosome (200-300S) peak. The 74S peak represents single inactive ribosomal units.

At present we are attempting to repeat these experiments utilizi g bacteria for these studies. Figure 3 represents results obtained when C^{14} uridine was added to 72 hour cultures of (1) axenic amoebae, (2) amoebae with virulence-inducing bacteria, and (3) amoeba with non-virulence-inducing bacteria. These experiments should be

looked upon with some reservation since amoebae had to be washed free of bacteria by repeated washings in buffer and low speed centrifugation. Recognizing this limitation, it is clear that almost immediately after adding E. coli (VEI) the rate of C^{14} uridine incorporation increases suggesting increased RNA synthesis. Lower rates of incorporation are found in axenic amoebae and when non-virulence-inducing bacteria (VE II) are added.

Since we were interested in knowing whether or not newly incorporated amino acids were localized in acid phosphatase we attempted to localize radioactivity into newly synthesized enzyme. Figure 4 illustrates the results of such a pilot experiment in which amoebae were incubated for 10, 30 and 120 minute intervals and then received a 1.5 minute pulse of C^{14}amino acid mixture. The reaction was stopped by pouring the amoebae over frozen Earle's buffer and the nuclei were separated from cytoplasm by douncing as described above. The cytoplasm was separated by disc electrophoresis and then was gel stained by the Gomori technic. The gel was next separated into 1 mm fractions in a Maizel gel fractionator and each fraction was read in a low-background scintillation counter. Our initial results are most encouraging and indicate that newly synthesized protein is acid phosphatase. These studies are continuing in order to confirm whether new protein synthesis is being made on newly synthesized RNA. It is hoped that these studies will disclose the means by which this cell controls virulence.

III. Ultrastructural Studies

In order to determine if changes in virulence are reflected in the ultrastructural cytology of E. histolytica we are studying each of our strains by means of electron microscopy. Furthermore, we have undertaken a series of experiments aimed at precise localization of the cellular sites of intracellular synthesis in E. histolytica.

Our initial studies have essentially confirmed observations by several other investigators (3, 4, 5). We have examined amoebae at various stages in their life cycle and find that there are no identifiable mitochondria, Golgi membranes or lysosomes; a well-defined endoplasmic reticulum is lacking. Moreover, one is able to demonstrate a well ordered or crystalline-like structure in most trophozoites. This chromatoid or ribonucleoprotein (RNP) material has been described by several workers including Siddiqui and Rudzenska (6) in E. invadens. The functional significance of this structure has remained unclear. Since we obtained evidence that RNA synthesis is related to the enhancement of virulence it was clearly indicated that we should carefully examine the RNP material of our experimental amoebae.

Although Barker and Svihla (7) clearly established the nucleic acid nature of the chromatoid (RNP) material in E. invadens, we felt unequivocal substantiation of these findings in E. histolytica was desireable. We, therefore, harvested a 96 hour axenic culture of Strain 301, fixed them in 2% glutaraldehyde for 2 hours, washed

them for six hours, and then infiltrated them with a water-soluble plastic embedment. Sections were next incubated in chromatographically pure ribonuclease (RNase) for up to 4 hours. These sections were stained and examined in the electron microscope. A study of these grids has revealed progressive digestion of the chromatoid material. There remains, however, an underlying membranous tubular structure which can best be described as microtubules. The significance of these tubules as other than supporting structure is now being considered.

In view of our observation that amoebae exposed to virulence-inducing strains of E. coli incorporate C^{14} uridine at a greater rate than axenic forms, trophozoites were examined after a 4 hour exposure to β-glycerophosphate. These sections indicated increased amounts of RNP material. Such observations appear to confirm our earlier impression. Radioautographic studies are incomplete at this time but do lend additional support to our working concept that RNA synthesis precedes protein synthesis. These events appear to be associated with a rise in virulence as measured by animal inoculation studies.

The evidence we have been accumulating appears to implicate the RNP body as a highly specialized dynamic structure which serves as the protein-synthetic site of E. histolytica (and probably other members of the genus). It is apparent that a great deal more remains to be done in order to clarify each step of the process controlling cellular virulence. The studies being reported here are only the initial steps that have provided us with a working concept of the pathobiology of E. histolytica.

IV. Axenic cultivation of several strains of Entamoeba histolytica in our laboratory has permitted study of various aspects of amoeba, bacteria, and host inter-relationships. Axenic cultures have allowed us to investigate aspects of the growth requirements of E. histolytica without the confusion and contamination from other associated organisms as has been the previous experience. Ultrastructural features of this organism have been studied by electron microscopy in order to ascertain possible structural differences between axenic and bacterized organisms. Attempts to correlate aspects of structure with some aspects of macromolecular synthetic mechanisms have also been undertaken using a variety of techniques.

Each part of our effort in this study is designed to provide a step towards further understanding of the cytophysiology of E. histolytica and to help unravel the puzzeling problems related to strain virulence and control of invasiveness.

During the past year, this laboratory devoted considerable effort to establish our axenic amoebae, previously grown in biphasic medium, into a monophasic medium. This attainment was considered most important prior to making any realistic effort to further define the culture requirements of E. histolytica . Further, we have continued our studies on the role of bacteria in amoebic virulence and the possible ultrastructural significance of the amoeba's cytoplasmic organelles to various life processes concerned with invasibility and virulence.

V. Improved Culture Methods

During the past year we have been able to improve our culture methods such that we can now grow E.histolytica in a monophasic non-defined medium (Fig 1). This medium and its basic preparation are described in detail elsewhere (Wittner,1968). Using this medium, growth is about equivalent to that in our best biphasic cultures (Fig 2) with some significant advantages. Monophasic medium has proven to be relatively easy to prepare; handling and harvesting of cells is far more convenient than with diphasic media. It should be emphasized that, especially for obtaining large numbers of organisms for biochemical studies, harvesting of cells without damage and in large numbers is an absolute requirement. Presently, serum remains an absolute requirement for sustained growth of the amoebae. Future plans call for electrophoretic separation of serum into multiple fractions in order to determine what factor or factors are essential for growth. If successful, this will be a large step toward a defined medium.

In order to initiate monophasic cultures it was necessary to inoculate between 40,000-60,000 amoebae/ml of medium. Usually growth was found to be excellent during the initial 3-5 transfers which were carried out at 72 hour intervals. The most critical period for adaptation in monophasic medium was between the 5th to 12th transfers. For reasons not yet fully understood, most cultures fail during this period. Establishment of the first monophasic cultures required 238 separate attempts. Since becoming established they have been sub-cultured every 6-7th day. Employment of Ionagar as recommended by Diamond (1968) has a modest salutory effect on initial growth but does not seem to effect the final number of amoebae attained after one week.

Presently, we are attempting to establish minimal levels of asamino acids, lactalbumin hydrolysate and trypticase and still aintain growth. Diminished levels of these constituents in the ıedium are desirable in order to achieve still "cleaner" preparations especially when cultures are pooled to acquire sufficient material for further biochemical definition of the various functional states of these amoebae.

VI. Studies on Mechanisms for Control of Virulence

a) Previously we showed that axenic amoebae inoculated into the caecum of germ-free guinea pigs or the liver of hamsters did not produce disease. However, when reassociated with suitable bacterial strains for as little as 12 hours, virulence is again encountered and severe caecal ulcerations or liver abcesses could be obtained. Utilizing a radiological assay method dependent on low-background scintillation spectrometry, we obtained evidence that reassociation of axenic amoebae with suitable bacteria is associated with a detectable rise in amoeba RNA synthesis within 30 min (see last Annual Report Fig 3). Experiments were next carried out to ascertain the bacterial factor (s) which might be responsible for these results. Initial experiments demonstrated living bacteria were ingested by amoebae within 5 minutes after exposure, as were bacteria previously killed by heat or X-radiation. However, ingestion of dead bacteria by axenic amoebae did not result in heightened virulence. Similarly, crude bacterial extracts were no more effective than dead, whole bacteria. With these data in view, Clostridium perfringens and Escherichia coli were grown in the presence of 20 uc/ml H^3-methyl-thymidine for 24 hours to ensure heavy incorporation into bacterial DNA (5). The bacteria

were then washed and the radioactivity of a standard aliquot of a 10% TCA precipitatable fraction determined in a low background scintillation spectrometer. The remaining washed and labelled bacteria were divided into three fractions (1) living (2) heat killed, and (3) X-radiation killed bacteria. Each fraction was then added to cultures of axenic amoebae. Every five minutes, aliquots were removed and subjected to repeated washing with ice cold saline until free bacteria could not be detected by phase microscope inspection. The amoebae were next placed in an ice-cold "swelling" (hypotonic) buffer for 10 minutes and then "dounced" with a specially designated homogenizer in order to separate nucleus from cytoplasm. This procedure together with subsequent washing and inspection under the phase microscope gave very clear separations of nuclei with few cytoplasmic tags. Radioactivity was determined for each fraction as described previously. Fig.3 indicates that there is progressive accumulation of radioactive label derived from living bacteria in the nucleus of the amoeba. In contrast, the radioactive label from previously killed bacteria fails to localize in the nucleus. Although not unequivocally proven by this experiment these results suggest a possible mechanism by which living bacteria influence virulence of amoebic strains. It would seem that bacterial DNA may play an important role in the virulence in E. histolytica. Just how such DNA acts to alter amoebic virulence can only be speculated upon at this time. It may, for example, serve to direct the increased synthesis of various enzyme proteins, such as described below. Presently we are repeating these studies utilizing a strain of Escherichia coli known to be associated with virulent strains of E. histolytica in order to determine whether or not we can obtain similar results with other

virulence associated bacteria. It seems likely, moreover, that the heat or X-radiation killed bacteria which proved unsuitable with regard to increasing the amoeba's virulence may be the result of destroying, denaturing, or inactivating DNA. Recently, we have performed repeated experiments in which crude bacterial extracts added to axenic cultures were found to be inactive with regard to enhancing amoebic virulence. Presently, we are preparing a bacterial DNA from C. perfringens in order to repeat the above experiments with a purified DNA recovered from a bacterial strain known to enhance virulence when placed with axenic amoebae. If this labelled DNA is found in the nucleus, we will proceed and inoculate such amoebae into germ-free animals.

High resolution electron microscopic radioautographs have also been prepared for these experiments but have not been fully evaluated at this time. It is anticipated that these radioautographs will localize the nuclear site of the incorporated radioactive label and should provide morphologic corroborration of the above described results at the ultrastructural level.

Experiments were also carried out in order to learn if bacterial RNA could be incorporated by axenic E.histolytica. In these studies, cultures of Clostridium perfringens and Escherichia coli were grown for 24 hours with 25 u c/ml H^3-uridine. The bacteria were harvested, washed repeatedly in saline, and the radioactivity of a standard aliquot of a 10% TCA precipitable fraction was determined, previously described. The remaining washed and labelled bacteria were again divided into 3 portions: 1) living, 2) heat killed, 3) X-ray killed and added to cultures

of axenic amoebae. The cultures were sampled with fractions taken at 5 minute intervals as previously described. The results can be seen in Fig. 4. It is clear from these data that little or no RNA enters the nucleus of the amoebae. The radioautographs for these experiments are not yet developed but they should show whether or not cytoplasmic label is localized to engulfed bacteria only or to other areas of the amoebae as well.

As we have suggested before, the RNP bodies appear to be a highly specialized form of endoplasmic reticulum and their role in overall as well as specific protein synthesis may be important, especia as concerns adaptive changes leading to alterations in the degree of amoebic virulence (3,4).

b) In our previous annual report we described increased hydrolytic enzyme synthesis in axenic amoebae exposed, for example to increased organic phosphosphates in the culture medium. Since we regarded this observation as a possible "model" for the increased synthetic processes that may take place in the axenic amoebae when they are exposed to bacteria we proceded to further study this system. We previously obtained data suggesting that such increased enzyme synthesis might be related to the RNP body and we thought it important to visualize the site of enzyme synthesis <u>in situ</u>. Since we have had most experience with acid phosphatase we decided to localize this enzyme by electron microscopic histochemical methods. Axenic cultures were exposed to 25 mg/ml glycerophosphate and samples were removed and processed for electron microscopic histochemistry after 1,3,6,9,

and 12 hours exposure to organic phosphate. Examination of electron micrographs revealed a progressive increase in phosphatase activity especially in the 1,3 and 6 hour samples. Beyond this period subjective observation of the cells did not reveal any further increase in enzyme activity. Of great interest, however, was the consistant observation that enzyme activity was found to be associated with filamentous structures at the cell surface and within vacuoles. These filaments have the approximate dimensions of the core material underlying the helical portion of the RNP bodies. In view of our previous experiments (see last Annual Report) where we were able to relate enzyme protein synthesis to the RNP body it would seem that the acid phosphatase that is synthesized on the RNP body must be present in an inactive or masked form, i.e. similar perhaps to a pepsinogen-pepsin system as defined in vertebrate systems. To relate a similar scheme to E. histolytica, we could postulate that the enzyme protein could be carried to the vacuole on the helical RNP bodies where they would then be"dumped" or released in the same way into the vacuoles for digestive purposes. In a sense these would then be serving as the lysosome-equivalent for Entamoeba histolytica. Synthesis of specific hydrolytic or proteolytic enzymes could also serve as a means of tissue digestion during invasion of host tissues.

This year, we have been repeating the above experiments by feeding axenic amoebae suitable strains of Clostridum perfringens in order to determine if enhanced synthesis occurs in a similar fashion to the model enzyme system. In these experiments we are attempting to localize ATPase, B-glucuronidase, thiamine pyrophosphatase, as well

as acid phosphatase. Further, we are expanding this work by developing other systems designed to challenge the ability of axenic trophozoites to synthesize various kinds of enzymic activity. If our concept that the helical RNP system of these organisms is a multi-potent, highly labile, protein synthetic system, then we can anticipate that it can utilize information provided by substrate, for example, to produce highly specific kinds of enzyme protein. In this way, the amoeba may alter its degree of virulence and change the extent of its invasive capacity by meeting the challenge of different substrates as these appear in the organisms environment in host tissue. If this does occur, it is of extreme interest and quite economical biologically in view of the scarcity of classical organelles in E. histolytica.

The experiments designed to test these various substrates are undertaken quite similarly to that described above for acid phosphatase stimulation. A relatively broad range of substrates are used, their selection dependent on the availability of a suitable enzyme assay system with which to test the synthetic capacity of the amoeba. Curren we are using as substrate various phosphates including adenosine, mono-di- and triphosphate, the mono-, di, and triphosphates of uridine and cytidine, thiamine pyrophosphate and creatinine phosphate. The test system for adaptibility of the amoebae to these substrates is a dual one using both cytochemical staining methods with the light and electro microscope and biochemical test system on washed aliquotes of homogenize

amoeba. Light microscope cytochemistry provides an initial screening to determine whether any detectable levels of enzyme activity are present in intact organisms. Based on these results, the biochemical assay is undertaken using the identical substrate employed to stimulate enzyme activity. Simultaneous with the light microscope study, material is prepared for electron cytochemistry. When this material is examined, if any detectable enzyme activity is present at the ultrastructural level, a second biochemical assay with increased sensitivity is run where ever possible. Using this test protocol, we have detected increased activity with all the triphosphated nucleosides as well as with thiamine pryophosphate. This increase is within the range of that previously determined using beta-glycerophosphate. There are also lower but distinctly increased activities with all monophosphate nucleosides but no apparent response to corresponding dinnucleosides. Creatinine phosphates do not increase levels of endogenous phosphatase activity in axenic amoeba. We have not as yet tested the ability of bacterized amoebae to respond to this test system although it is the next step in these studies. Also projected are use of a variety of other substrates, all of them likely candidates for increasing endogenous amoebic enzyme activity of specific type. These include several polysaccharides including mucin and glycogen, and sulfur-containing (SH) compounds. We are, in affect, attempting to reconstruct an <u>in vitro</u> system of pure substrates which might be similar to those the organism would have to deal with during invasion of host tissues <u>in vivo</u>. Once

we have understood the dynamics of the amoebas' response to such substrates, we shall then proceed to undertake a more detailed serie of experiments dealing with the adaptive response to such systems. This type of experiment may provide us with an understanding of the underlying basis of changing virulence so characteristic of strains of Entamoeba histolytica invading host tissues.

VII. In our last Progress Report we described improved culture methods such that we now grow Entamoeba histolytica (strains 301, F-22, and K-9) in monophasic medium. The basic preparation of this medium is described elsewhere (Wittner, 1968). The monophasic medium has proven relatively simple to prepare. The handling and harvesting of cells has been more convenient than with any of the biphasic media we have employed in the past. At present a four to twenty times increase is obtained over a 96 hour growth period. During the present investigative period we have undertaken studies to establish maximum and minimum serum protein requirements that will permit sustained growth of amoebae in monophasic medium. Less than 3.5% and more than 16.5% horse serum was found to be unsuitable for sustained cultivation.

In selecting serum for study of protein requirements of axenized amoebae, it became clear that bovine serum gave greater yields of amoebae in a 96 hour growth period than horse serum. We studied the efficacy of fetal calf, calf, bovine, porcine, as well as horse serum, with regard to their growth-promoting abilities. It was apparent almost immediately that fetal calf was unsuitable, and that bovine serum consistently gave higher yields of amoebae. Because of these observations, electrophoretic separation of bovine serum was initiated.

Utilizing preparative starch block electrophoresis, bovine serum was separated into eight fractions. Each fraction has been tested for its ability to support growth of E. histolytica. A globulin fraction containing a high concentration of β-lipoprotein was found to support growth of amoebae without other protein supplement to the monophasic medium. Subsequent analysis of this fraction, utilizing continuous elution on a QAE-Sephadex A-50 column with a tris-HCl buffer as eluant resolved three peaks. One of

these peaks has been found to possess the growth promoting factor in serum.

The active serum fraction is being studied intensively in order to identify it precisely. At this writing it is believed to be a phospho-lipid factor.

VIII. A. Ultrastructural and Autoradiographic Studies

As previously described, a characteristic ultrastructural feature of members of the genus Entamoeba is the presence of ribonucleoprotein in ordered helical arrays. These form the "chromatoid bodies" more evident in cysts, but also seen in trophozoites. Since these bodies, as well as shorter cytoplasmic helices, appear to represent the only organized form ribosomes assume in these amoebae, their detailed structure and function in relation to the economy of E. histolytica were of interest, especially with regard to pathogenicity. In addition, evidence has been accumulating that illustrates many other cell types are capable of producing ribosomal helices. Thus an understanding of how such structures may be produced, as well as the circumstances that induce ribosomal helices, was considered appropriate to our investigations.

Previously, we described some features of helical RNP structures in axenically grown and bacterized trophozoites of E. histolytica, stressing the presence of a "core" comprised of filamentous material. Such filaments were detected in cytoplasmic RNP helices following digestion with RNase. Similarly described was the presence of core material, apparently derived from helices in digestive vacuoles. Recently, we have observed detailed relationships between helical RNP bodies and their filamentous core. Moreover, we have found specific regions in the cytoplasm of trophozoites which appear to represent the ultrastructural sites for RNP helix formation.

The study of RNP body formation was made possible when it was noted that trophozoites of E. histolytica produced large numbers of RNP bodies following exposure to

Vinca alkaloids. Some increase was noted after 30 minutes, but by 4 hours the increase was unequivocal. Subsequently the formation of chromatoid bodies was traced by sampling cultures every 15 minutes during exposure to the alkaloid. Once the pattern was understood we could then follow the formation of RNP bodies in normal or untreated cells. In both untreated and alkaloid exposed trophozoites many glycogen-free areas could be detected throughout the cytoplasm. Many of these regions were composed of an amorphous, electron-dense material interspersed with numbers of free ribosomes. In some of these areas, short, randomly oriented filaments could be detected. Frequently, short helical fragments were also present in these areas. Further examination showed the presence of linear arrays of ribosomes in a form suggestive of polysomes, but which were not in the configuration characteristic of the larger helical bodies.

With longer periods of alkaloid exposure, many more glycogen-free regions composed of packed filaments and free ribosomes became evident. While some areas contained densely packed filaments, others frequently were associated also with stacked RNP helices such that both areas were intimately associated with one another. Scattered among the filaments were free, single ribosomes or chains of ribosomes. Other filament configurations were noted. While all the features of RNP body could be detected in normal cells, they were much more readily detected in alkaloid treated amoebae since the frequency of occurrence was much greater.

B. Association of Filaments and RNP Particles

Careful study of alkaloid treated amoebae by high resolution electron microscopy demonstrated the intimate relationship between filaments and RNP arrays. Filaments most frequently appeared to be composed of a duplex each of 60Å, upon which the helical RNP arrays are situated.

C. Autoradiography

Incorporation of labelled RNA precursors into glycogen-free, filamentous, ribosome-containing regions is clearly demonstrated by high resolution autoradiography Localization of labelled material depended on the length of exposure to the radioactive precursors. Short pulses of up to 30 minutes with H^3-uridine revealed incorporation solely in filament regions, whereas with a three hour pulse, labelling was found over packed RNP helices.

The observations presented in parts 2 A,B,C, provide further evidence for the presence of core material associated with ordered helical ribosomal arrays constituting the chromatoid bodies and the short cytoplasmic RNP helices in trophozoites in E. histolytica. Our earlier experiments (see last Annual Report) using RNase digestion of water-soluble methacrylate embedded trophozoites suggested the presence of a central core, the existence of which is consistent with the postulated "core" material in equivalent helical crystals of E. invadens (Morgan & Uzman, 1966) and HeLa cells (Weiss and Glover, 1968). Furthermore, our present studies unequivocally identify sites of helical RNP assembly in the cytoplasm of E. histolytica. Presently, we regard the regions of glycogen-free filament arrays as the site of chromatoid body assembly. Further evidence of assembly with m-RNA is under investigation at this time.

It is interesting to note that in addition to members of the genus Entamoeba, helical ribosome arrays have now been described from a variety of differentiating vertebrate cell types, plant cells, bacteria, cultured cells, and adult mammalian cells. The means whereby such helices are formed and maintained remain obscure. Work with Entamoeba may provide the clue to such structures. We currently regard these structures in E. histolytica as a highly labile equivalent to rough ER which, in the packed configuration

i. e. RNP body, may serve as a synthetic reserve. Previously, we presented evidence suggesting the relationship between short helical RNP bodies and digestive vacuoles. There then appears to be the release of 60Å core-derived protein into the vacuole. Thus the present evidence of sites of cytoplasmic ribosome filament assembly appears to represent initial stages in the production of a highly specialized synthetic system normally present in Entamoeba histolytica and modulated by the environment.

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